A STUDY OF CARBONMONOXIDE HEMOGLOBIN

PREPARATION, ANTIGENIC PROPERTIES, CRYSTALLOGRAPHY AND SPECTROGRAPHY

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ALDEN KINNEY BOOR

B.S., John B. Stetson University, 1914
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S.M., University of Chicago, 1923

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN PHYSIOLOGICAL CHEMISTRY IN THE GRADUATE SCHOOL OF THE UNIVERSITY OF ILLINOIS, 1930

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PREPARATION AND ANTIGENIC PROPERTIES OF CARBONMONOXIDE HEMOGLOBIN

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THE PREPARATION OF PURE CARBONMONOXIDE HEMOGLOBIN

Preparation of more or less pure oxyhemoglobin by methods suitable for use in large scale production was early recorded by Hoppe-Seyler,[1] Zinoffsky,[2] Hüfner[3] and others. These were essentially as follows: red corpuscles were laked with water, ether, or ether and alcohol and from the solution so obtained oxyhemoglobin was separated by crystallization in the cold from about 25% alcohol. Schulz[4] precipitated hemoglobin from laked red corpuscles with ammonium sulphate. It was not until after the beginning of the twentieth century, however, that methods were devised for the preparation of purer material. In most methods crystallization was relied upon for the purification of the hemoglobin, the P_H being controlled and regulated. Butterfield's[5] method is a modification of Hoppe-Seyler's. The method of Christian Bohr,[6] revised by Barcroft and Roberts,[7] and also by Barcroft and Hill,[8] is essentially as follows: Red corpuscles obtained by whipping and centrifugating dog blood were washed three times with Ringer's solution, laked with ether and the solution filtered through a Nutsche filter (Barcroft and Roberts) and dialyzed against ice water, or the corpuscles were placed directly into the dialyzer (Barcroft and Hill). The material was then concentrated by vacuum distillation at 40 C.

Dudley and Evans[9] prepared a saturated solution of reduced horse hemoglobin by dialysis of the corpuscles against distilled water at the osmotic pressure of hemoglobin. Then, by aeration the more insoluble oxyhemoglobin was formed, and, since it was somewhat less soluble than the reduced form, it crystallized out of solution. Heidelberger[10] passed a stream of gas composed of four parts CO_2 and one part O_2 through washed red corpuscles; this procedure insured the correct P_H (6.6 to 6.8). One-seventh volume toluene was added and the whole allowed to stand overnight in a refrigerator. Three layers appeared, the upper layer being almost entirely toluene and cell fragments, the middle layer a clear solution and the lower the crystalline oxyhemoglobin. Recrystallization was accomplished by dissolving in sodium carbonate solution and then readjusting the P_H with the CO_2 to O_2 mixture. This method is unsuitable for any but the more easily crystallizable hemoglobins such as that obtained from dog or horse blood. Haurowitz[11] dialyzed

Received for publication, Sept. 7, 1929.

1. Med. Chem. Untersuchungen, 1867, 2, p. 181.
2. Ztschr. f. Physiol. Chem., 1886, 10, p. 16.
3. Arch. f. Anat. & Physiol., Physiol. Abt., 1894, p. 130.
4. Ztschr. f. Physiol. Chem., 1898, 24, p. 449.
5. Ztschr. f. Physiol. Chem., 1909, 62, p. 173.
6. Skandin. Arch. f. Physiol., 1892, 3, p. 76.
7. J. Physiol., 1909-1910, 39, p. 143.
8. Ibid., p. 411.
9. Biochem. J., 1921, 15, p. 487.
10. J. Biol. Chem., 1922, 53, p. 31.
11. Ztschr. f. Physiol. Chem., 1924, 136, p. 147.

crude hemoglobin for two days, which was drastic treatment of this rather fragile protein. Parsons[12] centrifugated, during thawing, a previously frozen oxyhemoglobin solution, separating an upper layer of ice and lower layer of hemoglobin crystals. This is a suitable method if a rather pure hemoglobin solution of a readily crystallizable nature, as that from horse, is used. Using a simple electrodialysis apparatus, Stadie and Ross[13] prepared crystalline hemoglobin from horse and dog red cells, or a concentrated solution of hemoglobin from sheep or beef, very low in inorganic impurities. Ferry and Green[14] prepared what they considered to be pure horse oxyhemoglobin, by centrifugating horse blood and crystallizing at 2 C., from an acid solution, P_H 6.6. They adjusted the P_H by adding 160 cc. to 320 cc. 0.1 N-HCl per liter of concentrated solution laked corpuscles or 0.1 M phosphate buffer solution, redissolved by adding 1. N-KOH, and recrystallized by making acid again to P_H 6.6. Any local excess of acid or alkali denatures the hemoglobin immediately. In alkaline solution this protein is especially unstable. The purity of the product is therefore questionable.

Dudley and Evans[9] criticize the methods relying alone upon crystallization as follows, "although the crystals appear to be homogeneous, the analysis of oxyhemoglobin prepared by these methods, even from the same species, vary very considerably." Abderhalden's[15,16] elementary analysis of oxyhemoglobin shows that one crystallization may still leave as much as 15% of foreign protein with the hemoglobin although the crystals may appear to be regular and homogeneous.

The method which is much superior to any of these methods is that of Marshall and Welker.[17] These investigators found that oxyhemoglobin is the only protein of the many tested by them, and the only blood protein which aluminum hydroxide cream fails to remove from solution. The red corpuscles were separated by centrifugating the oxalated blood, washed three or four times with physiologic sodium chloride solution, and laked with distilled water. A sufficient quantity of a specially prepared aluminum hydroxide cream was added to remove all the proteins except oxyhemoglobin. This was approximately a volume equal to the laked blood and the sufficiency of the material was determined by the ease of filtration. A pure oxyhemoglobin solution filters readily. This was filtered in a refrigerator. The filtrate may be freed from the excess inorganic matter by dialyzing for a few hours against distilled water, or, in some cases, by crystallizing. This is a mild treatment, and if carefully and rapidly carried out, very little risk of denaturation appears probable. The aluminum hydroxide cream was prepared by adding a 1% solution of ammonium hydroxide to a 1% solution of ammonium aluminum sulphate slowly and with constant stirring until the reaction was just slightly alkaline. The precipitate formed was repeatedly washed by decantation until the supernatant liquid gave no test for ammonia with Nessler reagent.

This method is well recommended. Howard[18] after pointing out the fact that impurities occur in hemoglobin products of other methods, says, "This method is of such excellence as to warrant the opinion that much of the work on hemoglobin in the past in which discordant results have been obtained, might well be repeated using the method of Marshall and Welker for the preparation of the pure substance." Hunter and Borsook[19] recommend the method generously.

12. Proc. Fourth International Congress of Refrig., 1, p. 194.
13. J. Biol. Chem., 1926, 68, p. 229.
14. Ibid., 1929, 81, p. 175.
15. Ztschr. f. Physiol. Chem., 1903, 37, p. 484.
16. Lehrbuch der Physiologischen Chemie, part 1, 1914, p. 700.
17. J. Am. Chem. Soc., 1913, 35, p. 820.
18. J. Biol. Chem., 1920, 41, p. 537.
19. Ibid., 1923, 57, p. 507.

In the present work all hemoglobin derivatives were made from oxyhemoglobin prepared by the Marshall and Welker method. To further insure the fact that the material used was pure, crystallization was resorted to in the product of all species examined except human, but no evidence of further removal of protein or other improvement was obtained. However, this step appeared worth while, inasmuch as the largest portion of inorganic salts were thereby removed. The hemoglobin prepared as stated was carbonylated by saturating with CO gas generated by the addition of formic acid to warm, concentrated H_2SO_4. The gas was twice washed with NaOH followed by H_2O. This carbonmonoxide hemoglobin was kept at 0 C., or below, under an atmosphere of CO. Van Slyke and Hiller [20] have shown that a final tension over 20 mm. CO is sufficient to insure complete saturation of the hemoglobin. It, therefore, appears certain that with over 700 mm. CO tension no uncombined hemoglobin would be present.

Recrystallization from alcohol of both the oxyhemoglobin and carbonmonoxide hemoglobin appeared advisable. Absolute alcohol, redistilled from lime, and cooled to 0 C. was added to the oxyhemoglobin or carbonmonoxide hemoglobin at 0 C., 5% or less in the case of the rat oxyhemoglobin and carbonmonoxide hemoglobin, increasing with guinea-pig, horse, dog, turkey, chicken, duck, approximately in this order, to 38% for ox, sheep and hog carbonmonoxide hemoglobin. That from the rat was so insoluble that some crystals formed from 1% water solution at the low temperature used. The carbonmonoxide hemoglobin in every case was soluble in a more concentrated alcoholic solution than the oxyhemoglobin of the same species. Neither human oxyhemoglobin nor carbonmonoxide hemoglobin could be crystallized by this method. The well mixed solution was placed in the refrigerator at 0 C to —6 C. A special electric refrigerator was used. This refrigerator was built from a standard double icing unit and a box of single unit size. This arrangement made possible the maintenance of a temperature below 0 C. without unduly forcing the mechanism. After from one to five days crystals appeared. These were filtered off in the refrigerator and redissolved in ice water. Carbonmonoxide hemoglobin was now resaturated with CO gas. Whether oxyhemoglobin or carbonmonoxide hemoglobin, the material was recrystallized from alcohol at 0 C.

A water solution of carbonmonoxide hemoglobin, covered with CO and kept at 0 C. or below for a period of several months did not undergo

20. J. Biol. Chem., 1928, 78, p. 807.

any observable change. Some of the crystals were dried in a desiccator over H_2SO_4, under an atmosphere of CO, and 0 C. and were preserved under an atmosphere of CO in a refrigerator for a year with no apparent change. They were still completely soluble in water, gave carbonmonoxide hemoglobin spectrum when examined in the spectroscope, and remained red on addition of concentrated sodium hydroxide. Crystals were also kept at room temperature under carbonmonoxide for a few months and did not seem to change as indicated by the above tests. These crystals preserved at room temperature and exposed to air became darker red but remained clear. They were soluble in water. The spectrum indicated the presence of carbonmonoxide hemoglobin, oxyhemoglobin and a trace of methemoglobin.

When a water solution of oxyhemoglobin has been maintained at room temperature for a period of 24 hours a large part, if not all of the protein, has been modified, as the spectrum indicates. Spectrographs show clearly the difference in spectrum of fresh oxyhemoglobin and its brown dialysate, dialysis having been accomplished at room temperature. A report on the spectrographic work is to be published soon (Boor). Immunologically, no differences were manifested between oxyhemoglobin and its dialysate.

The fact that a water solution of carbonmonoxide hemoglobin is much more stable chemically then oxyhemoglobin when standing at room temperature was demonstrated by attempts to dialyze through a collodion bag. This was done under conditions similar to those described above with respect to oxyhemoglobin, except that in this case the material was kept saturated with carbonmonoxide by continually bubbling this gas through the liquids inside and outside the bag. Dialysis was continued for five weeks. In contrast to the case of oxyhemoglobin, biuret and precipitin tests showed no protein in this dialysate. The contents of the collodion bag still gave the carbonmonoxide hemoglobin spectrum.

CARBONMONOXIDE HEMOGLOBIN AS AN ANTIGEN

From the standpoint of the chemical aspect of immunology, it is of the greatest importance to know the effect of slight chemical changes of animal proteins on their antigenicity and species specificity. With this in mind the present immunologic work was begun. Since pure hemoglobin is now available, and since it has been conclusively proven that it is antigenic and mainly species specific, as discussed below, it was considered an appropriate protein with which to work. A pure carbon-

monoxide hemoglobin is easily made by the method described and is one of the most stable of hemoglobin derivatives when kept at 0 C. or below, under an atmosphere of sufficient CO tension. Even when kept at room temperature it is sufficiently stable to undergo dialysis. Considered as a conjugated protein, carbonmonoxide hemoglobin is a slightly chemically modified protein, although the modified portion is generally thought to be the nonprotein radical hematin. Confidence in the antigenicity of hemoglobin is essential for the present study. It has been variously reported by different workers as nonantigenic and antigenic.

Bordet,[21] Levene, [22] Ford and Halsey,[23] and Schmidt and Bennett [24] are among those investigators whose work seemed to indicate that hemoglobin is not an antigen.

Nolf [25] found positive results in 1900. Leblanc's [26] report in 1901 that beef hemoglobin is not only antigenic, but acts as a specific precipitinogen has been quoted (Hektoen and Schulhof [27]) as the real discovery. The fact that beef hemoglobin is a specific precipitinogen was confirmed by Ide [28] the next year. Another Frenchman, Demees,[29] reported positive results in 1907. Injection of watery extracts of red corpuscles into rabbits gave rise to specific precipitin antibodies according to the report of Klein [30] and of Hektoen and Schulhof.[27] The latter say, "While other antigens may be present in crude aqueous extracts of corpuscles, the main precipitinogen seems to be hemoglobin, which is shown to be species specific precipitinogen in confirmation of the early work of Ide and his pupils." In 1914 Bradley and Sansum [31] reported experiments on dog and beef hemoglobin. They found it antigenic with regard to its ability to produce anaphylaxis in sensitized guinea-pigs, and they also reported it species specific. Schmidt and Bennett,[24] who claim to have shown that hemoglobin is not antigenic, point out inaccuracies in this work, due to contamination of the material by serum proteins.

It was not until 1923 that evidence was presented which showed conclusively that hemoglobin is antigenic and immunologically species specific. In that year Hektoen and Schulhof,[32] Higashi [33] and Heidelberger and Landsteiner [34] reported results which, because of the purity of materials and high quality of technique used, proved conclusively that hemoglobin is antigenic. Hektoen and Schulhof [32] used hemoglobin prepared by the method of Marshall and Welker from the blood of eight different species of animals. This was the first time a reliably pure hemoglobin was used for the test. A definite species specificity was obtained with

21. Ann. de l'Inst. Pasteur, 1899, 13, p. 273.
22. J. Med. Research, 1904, 12, p. 191.
23. Ibid., 1904, 11, p. 403.
24. J. Infect. Dis., 1919, 25, p. 207.
25. Ann. de l'Inst. Pasteur, 1900, 14, p. 297.
26. La Cellule, 1902, 18, p. 335.
27. J. Infect. Dis., 1922, 31, p. 32.
28. La Cellule, 1904, 20, p. 261.
29. Ibid., 1908, 24, p. 421.
30. Centralbl. f. Bakteriol., 1, O., 1905, 39, pp. 303 and 438.
31. J. Biol. Chem., 1914, 18, p. 497.
32. J. Infect. Dis., 1923, 33, p. 224.
33. J. Biochem., 1923, 2, p. 315.
34. J. Exper. Med., 1923, 38, p. 561.

the one exception that a highly potent serum against beef hemoglobin reacts also to some extent with human and sheep hemoglobin. The precipitin test was used. Higashi,[33] using hemoglobin made by Hoppe-Seyler's method, a method by which a more impure material is prepared, found that it developed in the animal body precipitin, complement fixation and anaphylactic antibodies. He also showed that it is species specific. Heidelberger and Landsteiner,[34] using a hemoglobin material crystallized from ammonium sulphate solution found that serum, produced by immunization with it, reacts species-specifically with hemoglobin solutions. In the work of Hektoen and Schulhof [32] and that of Heidelberger and Landsteiner [34] a relationship in hemoglobins from related species is indicated. A little later Engelhardt [35] found that serum from rabbits immunized with dog hemoglobin gives a positive precipitin reaction.

Considerable discussion arose as to which portion of the hemoglobin molecule is responsible for the antigenicity. This property may be due to the molecule as a whole, or to the hematin, or the globin fraction. A globin solution was prepared by Schulz [4] by the action of just enough 0.1 N-HCl on hemoglobin solution at 37 C. to turn the solution brown in two or three hours. Then considerable hemin was extracted with an alcohol-ether mixture. The remaining solution, however, still contained hemin and perhaps hemoglobin. Attempts by Schmidt [36] and Gay and Robertson [37] to obtain a serum containing fixation antibodies from globin prepared by this method failed. Gay and Robertson [37] reported an apparent production of separate antibodies for globin and casein when globin caseinate is injected into rabbits. In 1920 Browning and Wilson [38] reported results which indicated that globin can act as a species specific antigen. Powerful complement fixation reactions depended on suitable quantitative relationships between antigen and antibody, and also, a suitable hydrogen ion concentration, just as Sachs and Altman [39] found in the case of the Wassermann reaction for syphilis. They claimed that antiglobin serum did not react with corresponding hemoglobin or serum proteins, thereby excluding the probability of contaminations due to poorly prepared globin solutions. Ishikawa and Sakurabayashi [40] claimed that the hemoglobin precipitin is formed exclusively from the protein part of hemoglobin. Hill and Holden [41] prepared a globin containing about 2% methemoglobin by hydrolyzing the laked, washed corpuscles with 2 N-HCl at a low temperature. They extracted with kieselguhr and ether at 0 C. Metaprotein was

35. Biochem. Ztschr., 1925, 163, p. 187.
36. Univ. California Publications in Pathology, 1916, 2, p. 182.
37. J. Exper. Med., 1913, 17, p. 535.
38. J. Immunol., 1920, 5, p. 417.
39. Ber. Klin. Wchnschr., 1908, 45, p. 699.
40. Tohoku J. Exper. Med., 1925, 6, p. 395.
41. Biochem. J., 1926, 20, p. 1326.

precipitated with $\frac{N}{2}$ NH_4OH and ether removed in a vacuum desiccator, still at 0 C. After dialyzing away salts at 0 C. a sample was dried over H_2SO_4 in the vacuum of a mercury pump. There is no doubt some basis to the claim of these authors that their product is less denatured than some others due to the low temperature used. The high concentration of acid and alkali used, however, may possibly modify the protein even when cold. It has not been shown that no part of the other corpuscular proteins are present with the globin. Wu [42] later modified Hill and Holden's method using methemoglobin instead of laked corpuscles and not such concentrated acid and alkali. He claimed a purer product. Hektoen and Schulhof [43] used a globin solution prepared by the Hill and Holden method in their study of hemoglobin antigenicity. From their experiments they conclude that "the antigenic group of hemoglobin forms a part of the molecule which may be split off by acids. It is probable that this antigen is globin, although the possibility of the presence of a third group is not yet excluded." Holden [44] found that injection of horse globin, prepared by the method of Hill and Holden, did not produce anaphylaxis in guinea-pigs. A protein, unlikely globin, and neither stroma nor serum protein, caused sensitivity of isolated uterus strip. Ottensooser and Strauss [45] claimed species specificity by precipitin and complement fixation reactions, having used a slightly alkaline globin solution. Hemoglobin also gave tests with the antiglobin serum. Their results are not as valuable as would be hoped for since the globin was prepared by a modification of the Schulz method from a commercial, dried hemoglobin preparation. Drying hemoglobin crystals causes denaturation as shown by Barcroft,[46] Bayliss (Barcroft: ref. 21) [46] and Van Slyke, Hastings, Heidelberger and Neill.[47] A large part of the work to date, on globin is unsatisfactory because no method assuring production of pure, undenatured globin has been reported.

Reichert and Brown [48] in their studies of hemin crystal say, "As far as this work of comparing the angles of the crystals from the different species, and of different substances from the same species, has gone, it clearly indicates that partial or complete isomorphism of all the hemo-

42. Proc. Soc. Exper. Biol. & Med., 1929, 26, p. 741.
43. J. Infect. Dis., 1927, 41, p. 476.
44. Australian J. Exper. Biol. & M. Sc., 1928, 5, p. 285.
45. Biochem. Ztschr., 1928, 193, p. 426.
46. The respiratory function of the blood, part 2, 1928, p. 60.
47. J. Biol. Chem., 1922, 54, p. 481.
48. The Carnegie Institution of Washington, Publication No. 116, 1909, p. 329.

globins, and also their isomorphism with the triclinic hemin." They examined the hemin crystals from a number of species of animals. They further stated [49] that conclusive evidence is at hand that hemin crystals show species specificity.

Attempts to obtain antiserum to hemin or hematin by injecting eight rabbits with a slightly alkaline (Na_2CO_3) solution of hemin proved unsuccessful. The hemin was prepared from oxblood by the method of Willstätter and Fischer.[50] Injection of rabbits, preparation of serum and precipitin tests were carried out exactly as with the hemoglobin and carbonmonoxide hemoglobin, description of which is found below. The hemin solution proved to be very toxic. Of the eight rabbits used, six died before the series of injections was completed and blood was drawn. The serum from the two rabbits which survived the series of injections did not give positive precipitin tests when tested with hemin solution of concentrations, 1 : 100, 1 : 1,000, 1 : 10,000, 1 : 100,000 and 1 : 1,000,000.

Hektoen and Schulhof,[32] and Heidelberger and Landsteiner [34] have reported that hemoglobin antiserum gave positive reactions against the homologous carbonmonoxide hemoglobin. Antigenicity and species specificity of oxyhemoglobins and carbonmonoxide hemoglobins were investigated in the work now being reported.

Antiserums were prepared from rabbits by five intravenous injections of increasing quantities (2 cc., 4 cc., 6 cc., 8 cc., 10 cc.) of a 1% solution of the antigen, at 4-day intervals. A sample of blood was drawn from the left ventricle of the heart four days after the last injection. After standing in the refrigerator overnight, the serum was poured off from the clot and centrifugated to separate free, suspended, red corpuscles. The precipitin test with this serum was carried out by stratifying approximately 1/10 cc. serum under 1/10 cc. solution of antigen in physiological salt solution. Antigen concentrations of 1 : 100, 1 : 1,000, 1 : 10,000, 1 : 100,000, 1 : 1,000,000, and 1 : 10,000,000, were used. Vials about 4 mm. inside diameter were used as precipitin tubes. Appearance of a distinct cloudy band at layer of contact of the two liquids after standing one hour at room temperature was the basis of a positive test. When the first sample of a serum gave a poor or negative precipitin test, as determined by the least concentration of antigen required, more injections were made, using 8 cc., 10 cc., and 12 cc. of the same solution of antigen as previously used. In some cases the titer of precipitin antibodies was greatly increased by the added injections.

Antiserums were obtained by injections of carbonmonoxide hemoglobin made from the blood of man, ox, sheep, hog, dog, turkey, rat, horse, duck, chicken and guinea-pig and from the oxyhemoglobins

49. Private communication, E. T. Reichert to Wm. H. Welker, and E. T. Reichert to A. K. Boor.

50. Ztschr. f. physiol. Chem., 1913, 87, p. 423.

from horse, hog, ox, sheep, dog, turkey, and guinea-pig. They were preserved in the refrigerator, with a trace of chloroform (Merk, reagent quality). Collection of these antiserums was carried on over a period of about a year. Instability of antibody substance is shown by the fact that by the end of this time some antiserums obtained early in the period had lost their activity. Fresh material was prepared in these cases. Although the dilutions of antigen giving positive tests ran as high as 1 : 1,000,000 in final reactions indicated on the table, in the earlier preliminary tests, made soon after the antiserums were prepared, titers as high as 1 :10,000,000 were not uncommon. When prepared and stored in the refrigerator, each antiserum gave a distinctly positive test on dilution of at least 1 : 100,000 of antigen injected.

Each antiserum was tested against the carbonmonoxide hemoglobin of all the species studied and against the oxyhemoglobins of all except duck and human. As the table indicates, each antiserum of a carbonmonoxide hemoglobin gave positive test not only against that carbonmonoxide hemoglobin, but, likewise against the oxyhemoglobin of the same species. The antiserum of an oxyhemoglobin was also active against the corresponding carbonmonoxide hemoglobin. Antiserums of the carbonmonoxide hemoglobin and oxyhemoglobin of the fowl, chicken, duck and turkey are not individually specific within this group, but only in so far as species other than this group are concerned.

Sheep carbonmonoxide hemoglobin antiserum gave a positive reaction against ox oxyhemoglobin when undiluted, but when the serum was diluted 1 : 3 with physiologic salt solution, this reaction disappeared, although the titer against sheep carbonmonoxide hemoglobin and sheep oxyhemoglobin was not appreciably changed. A relationship between hemoglobin of ox and sheep was noted also by Hektoen and Schulhof.[32] Dog carbonmonoxide hemoglobin antiserum (a) was specific for dog carbonmonoxide hemoglobin and dog oxyhemoglobin. However, another sample of blood (b) was drawn from the same rabbit after reinjecting with the dog carbonmonoxide hemoglobin. This gave positive tests with human, ox, sheep, rat and horse carbonmonoxide hemoglobin, and horse and ox oxyhemoglobin, as well as dog carbonmonoxide hemoglobin and oxyhemoglobin. As in the case of the sheep carbonmonoxide hemoglobin antiserum, when dog carbonmonoxide hemoglobin antiserum was diluted 1 :3 with physiologic salt, it (c) also became species-specific. The irregularities shown by the sheep and dog carbonmonoxide hemoglobin antiserum indicate the possibility of some common factor in the hemoglobin molecule which has only weak antigenic power.

TITER OF OXYHEMOGLOBIN AND CARBONMONOXIDE HEMOGLOBIN ANTISERUMS

Hemoglobins		Antiserums: Human COHb 1	Ox COHb 2	Sheep COHb 3	COHb Hog 4	Dog COHb(a) 5	Turkey COHb 6	Rat COHb 7	Horse COHb 8	Duck COHb 9	Chicken COHb 10	Guinea-Pig COHb 11	Horse HbO_2 12	Hog HbO_2 13	Ox HbO_2 14	Sheep HbO_2 15	Dog HbO_2 16	Turkey HbO_2 17	Guinea-Pig HbO_2 18	Dog COHb(b) 19	Dog COHb(c) 20
Human	COHb 1	++++	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+++	0
Ox	COHb 2	0	++	+++	0	0	0	0	0	0	0	0	0	0	++++	0	0	0	0	+++	0
Sheep	COHb 3	0	0	++++	0	0	0	0	0	0	0	0	0	0	0	++++	0	0	0	++	0
Hog	COHb 4	0	0	0	++++	0	0	0	0	0	0	0	0	++++	0	0	0	0	0	0	0
Dog	COHb 5	0	0	0	0	++++	0	0	0	0	0	0	0	0	0	0	++++	0	0	++++	++++
Turkey	COHb 6	0	0	0	0	0	++++	0	0	++++	++++	0	0	0	0	0	0	++++	0	0	0
Rat	COHb 7	0	0	0	0	0	0	++++	0	0	0	0	0	0	0	0	0	0	0	++	0
Horse	COHb 8	0	0	0	0	0	0	0	++++	0	0	0	++++	0	0	0	0	0	0	+++	0
Duck	COHb 9	0	0	0	0	0	++++	0	0	++++	++++	0	0	0	0	0	0	++++	0	0	0
Chicken	COHb 10	0	0	0	0	0	++++	0	0	+++++	++++	0	0	0	0	0	+++++	0	0	0	0
Guinea-pig	COHb 11	0	0	0	0	0	0	0	0	0	0	++++	0	0	0	0	0	0	++++++	0	0
Horse	HbO_2 12	0	0	0	0	0	0	0	++++	0	0	0	++++	0	0	0	0	0	0	+++	0
Hog	HbO_2 13	0	0	0	++++	0	0	0	0	0	0	0	0	++++	0	0	0	0	0	0	0
Ox	HbO_2 14	0	+++++	++++ 0(1-3)	0	0	0	0	0	0	0	0	0	0	++++	0	0	0	0	++++	0
Sheep	HbO_2 15	0	0	++++	0	0	0	0	0	0	0	0	0	0	0	++++	0	0	0	0	0
Dog	HbO_2 16	0	0	0	0	++++	0	0	0	0	0	0	0	0	0	0	++++	0	0	++++	++++
Turkey	HbO_2 17	0	0	0	0	0	++++	0	0	++++	++++	0	0	0	0	0	0	++++	0	0	0
Guinea-pig	HbO_2 18	0	0	0	0	0	0	0	0	0	0	++++	0	0	0	0	0	0	++++++	0	0
Rat	HbO_2 19	0	0	0	0	0	0	++++	0	0	0	0	0	0	0	0	0	0	0	0	0
Chicken	HbO_2 20	0	0	0	0	0	++++	0	0	++++	++++	0	0	0	0	0	0	++++	0	0	0

COHb = carbonmonoxide hemoglobin; HbO_2 = oxyhemoglobin; 0 = no reaction. Positive reaction indicated by the following: ++ = 100 to 1,000; +++ = 1,000 to 10,000; ++++ = 10,000 to 100,000; +++++ = 100,000 to 1,000,000; ++++++ = 1,000,000+ titer.

SUMMARY

Pure carbonmonoxide hemoglobin was prepared by treating oxyhemoglobin, made by the method of Marshall and Welker, with pure carbonmonoxide, and in all species used, except man, the carbonmonoxide hemoglobin was recrystallized from alcohol three or four times at 0 C. The watery solutions or dried crystals were preserved at 0 C. and at room temperature under an atmosphere of carbonmonoxide. The report of previous investigators that oxyhemoglobin is antigenic and mainly species-specific is confirmed by the precipitin test, and likewise carbonmonoxide hemoglobin was found to be antigenic and mainly species-specific. Cross reactions appeared in the case of closely related species, as duck, chicken and turkey, beef and sheep. Cross reactions of one dog antiserum and one sheep antiserum with other species were observed. These antiserums became species-specific when diluted 1 : 3, which suggests a common radical in the antigen having weaker antigenic powers than the species-specific portion of the molecule. This point requires further work.

A CRYSTALLOGRAPHIC STUDY OF PURE CARBONMONOXIDE HEMOGLOBIN

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PLATES 3 TO 5

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In their treatise on the crystallography of hemoglobin Reichert and Brown[1] recorded literature representing the history of hemoglobin crystallization to the date of its publication, 1909. Hünefeld[2] was given credit for being the discoverer of crystals of hemoglobin in 1840. K. E. Reichert[3] might be called the true discoverer of the oxyhemoglobin crystals, however, for his discovery is the actual foundation of our knowledge of hemoglobin. Six hours after the death of a guinea pig he found crystals on the maternal uterus and in the placentas of all four fetuses present. Funke[4] was said to be the first to prepare "blood crystals," 1851. They quoted Preyer's[5] monograph, "Die Blutkrystalle," as "the leading authority on the crystallography of hemoglobin" up to that time. Preyer[6] classified some biological species he studied according to the crystal system of the oxyhemoglobin crystals. But by far the most comprehensive monograph on the subject published up to the present time is that of Reichert and

[1] Reichert, Edward Tyson, and Brown, Amos Peaslee, The differentiation and specificity of corresponding proteins and other vital substances in relation to biological classification and organic evolution. The crystallography of hemoglobins. *The Carnegie Institution of Washington, Publication No. 116*, 1909.

[2] Hünefeld, F. L., Die Chemismus in der thierischen Organization, Leipzig, 1840, 160 pages.

[3] Reichert, K. E., *Arch. Anat., Physiol. und wissensch. Med.*, 1849, 197.

[4] Funke, Otto, *Ztschr. f. rat. Med.*, N. F. 1851, **1**, 172.

[5] Preyer, W., Die Blutkrystalle, Jena, 1871.

[6] Preyer, W., *Pflüger's Arch. f. d. ges. Physiol.*, 1868, **1**, 395–454.

Brown.[1] This memoir on the crystallography of hemoglobin refers particularly to the relationships occurring in its biological and crystallographical classifications.

They concluded that genus and species specificity are indicated by hemoglobin crystal structure. However, improvements may be made in the method of crystal preparation. In no case reported in that treatise did the investigators use pure compounds in the crystal preparations. Most of the crystals were prepared for microscopical observation as follows:

> The blood, if available in small quantity only, was dropped on a microscopic slide, laked with a drop or two of water or ether, ammonium sulfate or oxalate added, and the material allowed to concentrate by evaporation. Then it was covered with a cover-glass, and this sealed with balsam to insure only slow evaporation. Crystals appeared when kept in the refrigerator for from a few hours to months. If the material was available in sufficiently large quantities, the red corpuscles were settled from the oxalated or defibrinated blood by centrifuging, laked with ether, saturated with ammonium oxalate, centrifugalized, and crystallized on a microscopic slide, with the cover glass sealed with balsam. When crystallization was too rapid a retardant was used, such as plasma, egg white, etc. When crystallization did not take place at a satisfactory speed, the workers used reduced hemoglobin or carbonmonoxide hemoglobin. Methemoglobin and metoxyhemoglobin were crystallized in some cases. Blood which had stood for several days was sometimes used, often being clotted and even putrified.

It is suggested that the various organic impurities might have influenced the crystal habit, and even the crystal system and form. There is no evidence indicating that hemoglobin and plasma proteins or egg white proteins or stroma proteins do not form chemical combinations. If such a combination were present the crystals were those of this complex material, rather than of the pure hemoglobin derivative, as the authors quoted indicated. Crystal shape may have been influenced by occluded proteins from stroma and serum. When carbonmonoxide hemoglobin was desired the blood or corpuscular paste was saturated with illuminating gas. Of course this was not pure carbonmonoxide, and it is possible that derivatives other than carbonmonoxide hemoglobin were produced.

The oxyhemoglobins and carbonmonoxide hemoglobins, crystal photographs of which are appended, were carefully prepared by the

method described elsewhere.[7] This method was essentially as follows:

Oxyhemoglobin was prepared by the method of Marshall and Welker.[8] Red corpuscles were separated by centrifuging, washed with saline solution, and laked with water. The solution was mixed with aluminum cream and filtered in a refrigerator. The filtrate contained no protein other than oxyhemoglobin as was shown by Marshall and Welker in 1913. The aluminum cream was prepared by adding slowly, with constant stirring, a 1 per cent solution of ammonium hydroxide to a 1 per cent solution of ammonium aluminum sulfate until the reaction was slightly alkaline. The precipitate formed was repeatedly washed by decantation until the supernatant liquid gave no test for ammonia with Nessler reagent. Carbonmonoxide hemoglobin was prepared by saturating oxyhemoglobin with CO gas generated by warm formic acid and concentrated H_2SO_4. The gas was twice washed with NaOH and H_2O. The oxyhemoglobins and carbonmonoxide hemoglobins were recrystallized from absolute alcohol distilled from lime, at 0°C. or below. The amount of alcohol required for crystallization was 5 per cent or less in the case of the rat oxyhemoglobin and carbonmonoxide hemoglobin, increasing with guinea pig, horse, dog, turkey, chicken, approximately in this order to 38 per cent for ox, sheep and hog carbonmonoxide hemoglobin.

These oxyhemoglobins and carbonmonoxide hemoglobins were presumably pure chemical compounds, although in a few cases crystals of two varieties may have formed from the same solution, *e.g.*, chicken carbonmonoxide hemoglobin, dog oxyhemoglobin and hog carbonmonoxide hemoglobin. In several preparations, as horse oxyhemoglobin and carbonmonoxide hemoglobin, and dog and rat oxyhemoglobin, Reichert and Brown found varieties of crystals which they designated as α and β hemoglobin or carbonmonoxide hemoglobin. Bohr[9] had previously claimed that more than one kind of hemoglobin may occur in the same animal. In the work now being reported only one kind of crystal of horse oxyhemogobin and of horse carbonmonoxide hemoglobin appeared. Dog oxyhemoglobin may be represented by two forms, but only one form can be detected in dog carbonmonoxide hemoglobin. The brown rat β oxyhemoglobin, so-called by Reichert and Brown, may be according to them an isomer or

[7] Boor, Alden K., and Hektoen, Ludvig, *J. Inf. Dis.*, 1930, **46**, 1.
[8] Marshall, J., and Welker, W. H., *J. Am. Chem. Soc.*, 1913, **35**, 820.
[9] Bohr, Christian, *Skandin. Arch. f. Physiol.*, 1892, **3**, 76.

mimetic twin of the α form. No β oxyhemoglobin crystal from rat was observed in this work. Only one crystal form of rat carbonmonoxide hemoglobin appeared. Reichert and Brown only report one chicken oxyhemoglobin, although they say that they "sometimes look tetragonal but are orthorhombic according to optical characters." Figs. 20 and 21 show two kinds of crystals of chicken carbonmonoxide hemoglobin. Whether these belong to different systems is a question. Optical character was not investigated, but it is possible that the observation of Reichert and Brown holds also for the crystals of Figs. 20 and 21. Every case of dual form is questionable: the crystals investigated in this work suggest one hemoglobin per species.

Photomicrographs of crystals of carbonmonoxide hemoglobin of ox, sheep, hog, dog, turkey, rat, horse, chicken and guinea pig, and oxyhemoglobin of horse, hog, ox, sheep, dog, guinea pig and rat are shown. The photomicrographs were taken outdoors, in winter, when the temperature was below 0°C., using a Zeiss photomicroscope. No attempt was made to make a thorough study of the crystals at the time of photographing. All observations and conclusions were based on a study of the photographs.

Ox

Reichert and Brown described their ox oxyhemoglobin crystals essentially as follows: prismatic, belonging to the orthorhombic system and about four times as long as the width. Twinning was reported.

In Figs. 1 and 2 there seems to be one form, evidently long, lath-shaped, prismatic crystals, probably belonging to the orthorhombic system. Much longer, thinner crystals were obtained than those described by Reichert and Brown. Twinning is also observed.

Figs. 3 and 4 represent ox carbonmonoxide hemoglobin crystals which seem to be orthorhombic and appear much like the bison oxyhemoglobin crystals described by Reichert and Brown as orthorhombic. The brachydome angle of 125° measured by them is the same as angle of brachydome of bullock or ox oxyhemoglobin crystals observed by them.

Sheep

The preparation of sheep oxyhemoglobin was crystallized by Reichert and Brown at room temperature, some crystals being obtained within 5 hours of making the preparation. That represented by appended photographs was rather difficult to crystallize. Reichert and Brown report that needles, without definite outline, tapering to a point at either end, formed at first, but soon tabular crystals consisting of the base with a very short prism began to appear. Then after about a day, long, prismatic crystals appeared consisting of three pinacoids, elongated parallel to the vertical axis and generally flattened on the orthopinacoid.

On Figs. 5 and 6 no needles and no tabular crystals of type noted by Reichert and Brown are seen. All crystals are trapezoidal shaped: perhaps all are the "horse-type twins" of Reichert and Brown, these twins being united along a prism-base edge. Both penetrating and contact twins are illustrated.

The carbonmonoxide hemoglobin crystals of Figs. 7 and 8 are tabular entirely, being thin and fragile. They are orthorhombic in general although perhaps one shown is tetragonal.

Hog

Reichert and Brown report hog oxyhemoglobin crystals of the orthorhombic system, of short prismatic habit; twinning was not observed.

Figs. 9 and 10 indicate a thin, fragile, tabular form crystal with base almost square, and also, lathlike platelets about ten times as long as wide with rectangular ends. No crystals of the shape indicated by Reichert and Brown appear. Twins are present, being noticeable on some tabular form crystals.

On Figs. 11, 12, and 13 prismatic crystals of tabular form belonging to the monoclinic system appear. These are hog carbonmonoxide hemoglobin crystals. The axis perpendicular to the base is very short with respect to the other two. The four edges of the base are almost, if not quite equal. Twins of tufts and sheaf-like aggregates resemble Reichert and Brown's crystals of reduced hemoglobin.

Dog

Reichert and Brown's preparations of dog oxyhemoglobin were made as follows: it was prepared from whole blood "defibrinated by beating, and from blood kept liquid by oxalating, and also from mixtures of whole blood and blood plasma. The blood, either defibrinated or oxalated, was laked with ether and centrifugalized; and from the clear solution thus obtained, with or without the addition of plasma, the slide preparations were made." They found crystals of two types—orthorhombic which they called α oxyhemoglobin, and monoclinic which was designated as β oxyhemoglobin. Crystals of β oxyhemoglobin were only occasionally observed by them, "in perhaps one out of a dozen slides" and appeared to develop more readily in the blood to which no oxalate had been added.

In Figs. 14 and 15 are also found crystals which resemble the orthorhombic α oxyhemoglobin and the monoclinic β oxyhemoglobin of Reichert and Brown. The first type is evidently rectangular while the second has a base of rhombus shape which perhaps occur in trapezoidal "horse-type twins." As in the work cited above there are many more of the α oxyhemoglobin type than of the β type.

Figs. 16 and 17 show dog carbonmonoxide hemoglobin crystals of only one type. They are prismatic, orthorhombic and are generally shorter than the oxyhemoglobin, the ratio of length to the thickness varying from about 2:1 to 5:1.

Turkey

Turkey oxyhemoglobin crystals were not photographed.

Turkey carbonmonoxide hemoglobin crystals, Figs. 18 and 19, are probably of one variety, being tetragonal or orthorhombic, with the base edge about three times the vertical edge. Another form of crystal may be present, but this questionable figure is probably a plate on edge; the length is about three times the thickness.

Chicken

The chicken carbonmonoxide hemoglobin, Figs. 20 and 21, show crystals similar to those of Reichert and Brown found in the case of chicken oxyhemoglobin. They found orthorhombic crystals which "sometimes look tetragonal but are orthorhombic according to optic

characters." In both cases square tabular crystals occurred. Reichert and Brown's crystals aggregated into groups by piling up of the plates, or perhaps twinning on an axis normal to an edge (110–001); also by what appeared to be twinning on a dome, the crystals usually occurring in isolated clusters. On Figs. 20 and 21 some twinning on edge may be seen. The second kind of crystal is prismatic, with length about ten times the width.

Rat

Reichert and Brown examined oxyhemoglobin from four kinds of rats, white, brown, black and Alexandrine. Slight differences were found in the crystals from the Norway and albino rat on the one hand, and the Alexandrine and black rat on the other hand. In all four cases, however, orthorhombic crystals were found. These were prismatic, elongated, six-sided plates. A β oxyhemoglobin was found in the blood of the brown rat. It was isotropic and apparently isometric with the α oxyhemoglobin, but showed hexagonal outlines.

The material from which crystals of Figs. 22, 23, 24, and 25 were made was obtained from wild rats which, because of color variegations were thought to be crossed from white, black and Norway or brown varieties. Only one kind of oxyhemoglobin and one of carbonmonoxide hemoglobin was observed. Both appear to be elongated, hexagonal, tabular plates. The ratio of width to length is 1:5. They are both thin and fragile. The oxyhemoglobin in the accompanying photograph appears to be monoclinic.

The rat carbonmonoxide hemoglobin crystals, Figs. 24 and 25, are also elongated, hexagonal plates of tabular form. However, they probably belong to the orthorhombic system. They are about twice as long as the width. On Figs. 24 and 25 the original facial angles have been somewhat destroyed by exposure of the crystals to temperatures high enough to partially melt the edges.

Horse

Orthorhombic α oxyhemoglobin and α carbonmonoxide hemoglobin and β oxyhemoglobin and β carbonmonoxide hemoglobin crystals were obtained by Reichert and Brown from horse red corpuscles. Only one kind can be distinguished on photographs of pure horse

oxyhemoglobin (Figs. 26 and 27) and pure carbonmonoxide hemoglobin (Figs. 28, 29, and 30). The oxyhemoglobin may have the form of Reichert and Brown's oxyhemoglobin, appearing to be monoclinic prisms and all being fishtail, gypsum-type twins. The horse carbonmonoxide hemoglobin, on the other hand, seems to resemble the oxyhemoglobin in form, appearing to be tabular, orthorhombic crystals. Reichert and Brown report that long, hair-like needles form first which soon become more or less stout prisms. The length-width ratio of crystals vary from 8:1 to 50:1 for carbonmonoxide hemoglobin, and 10:1 in the case of oxyhemoglobin.

Guinea pig

Crystals of guinea-pig oxyhemoglobin and carbonmonoxide hemoglobin formed very easily. Orthorhombic, sphenoidal crystals appeared in both cases, as was reported by Reichert and Brown in the case of oxyhemoglobin. Most of the crystals on Photographs 31, 32, 33, and 34 seem to be right-handed sphenoid with corners truncated by left-handed sphenoid.

SUMMARY AND CONCLUSIONS

1. Photomicrographs of crystals of pure carbonmonoxide hemoglobin of the following species are presented; ox, sheep, hog, dog, turkey, rat, horse, chicken and guinea pig. Photomicrographs of the oxyhemoglobin crystals of the following species are also shown: ox, sheep, hog, dog, rat, horse and guinea pig. The crystals were formed from the pure protein by adding a suitable amount of ethyl alcohol and maintaining a temperature of 0°C., or lower.

2. In some species a sufficient difference is shown between the carbonmonoxide hemoglobin and oxyhemoglobin crystals to distinguish these compounds, but the photographs of crystals of carbonmonoxide hemoglobin and oxyhemoglobin of some species, such as guinea pig, show no appreciable difference.

3. Differences between the carbonmonoxide hemoglobins, as well as between the oxyhemoglobins, of the different species studied are indicated.

4. The carbonmonoxide hemoglobin crystals from the bloods studied are species specific in their nature, and, in many cases, can be dis-

tinguished from the analogous oxyhemoglobin by crystallographic study.

The author wishes to express his thanks and appreciation to Dr. William H. Welker, at whose suggestion this investigation was undertaken, for his kind advice, valuable assistance and inspiring encouragement in all of the work represented by this paper.

EXPLANATION OF PLATES

Plate 3

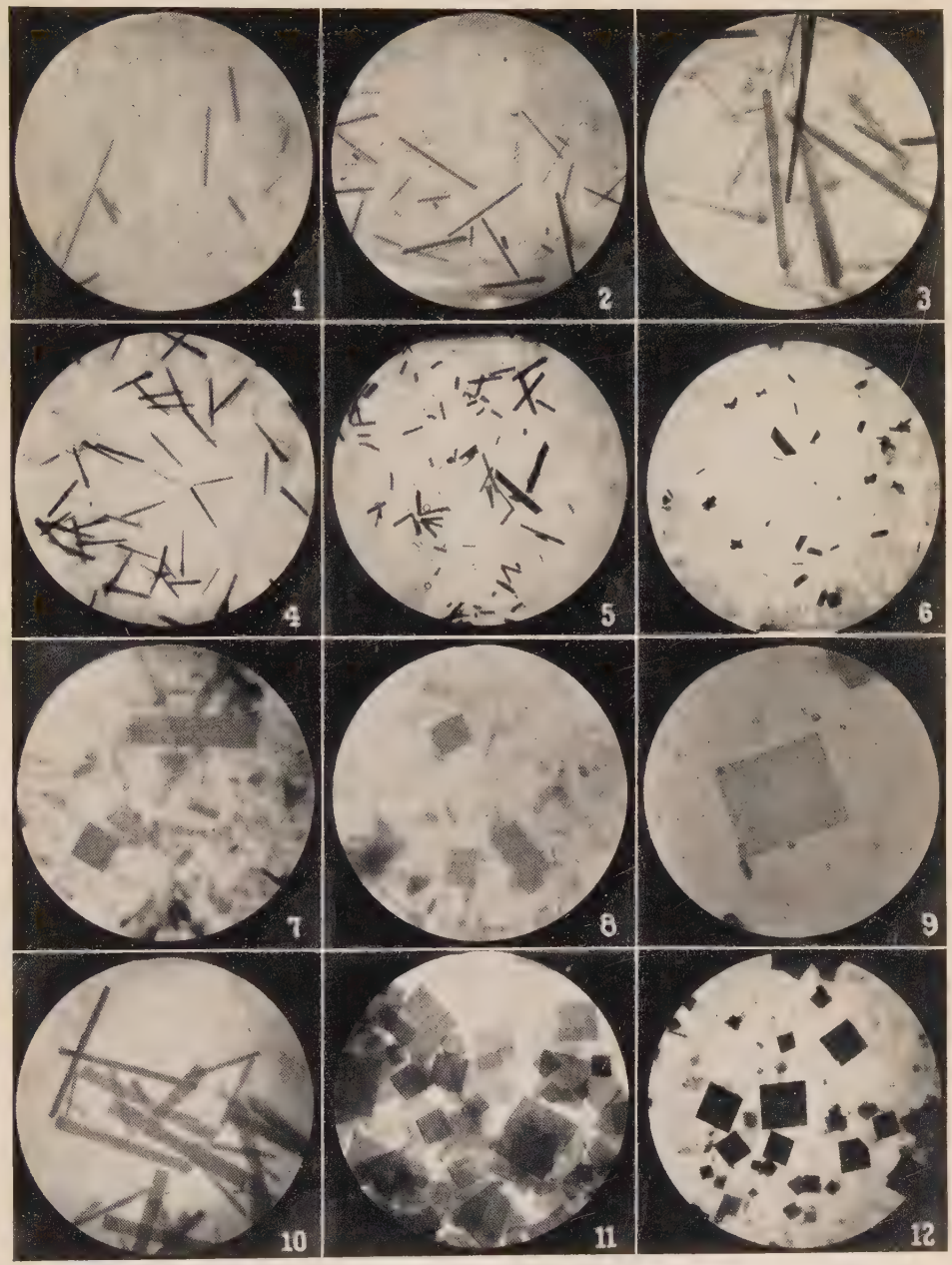
Fig. 1. Ox oxyhemoglobin. × 18.6.
Fig. 2. Ox oxyhemoglobin. × 18.6.
Fig. 3. Ox carbonmonoxide hemoglobin. × 62.
Fig. 4. Ox carbonmonoxide hemoglobin. × 18.6.
Fig. 5. Sheep oxyhemoglobin. × 18.6.
Fig. 6. Sheep oxyhemoglobin. × 18.6.
Fig. 7. Sheep carbonmonoxide hemoglobin. × 62.
Fig. 8. Sheep carbonmonoxide hemoglobin. × 62.
Fig. 9. Hog oxyhemoglobin. × 246.
Fig. 10. Hog oxyhemoglobin. × 62.
Fig. 11. Hog carbonmonoxide hemoglobin. × 62.
Fig. 12. Hog carbonmonoxide hemoglobin. × 62.

Plate 4

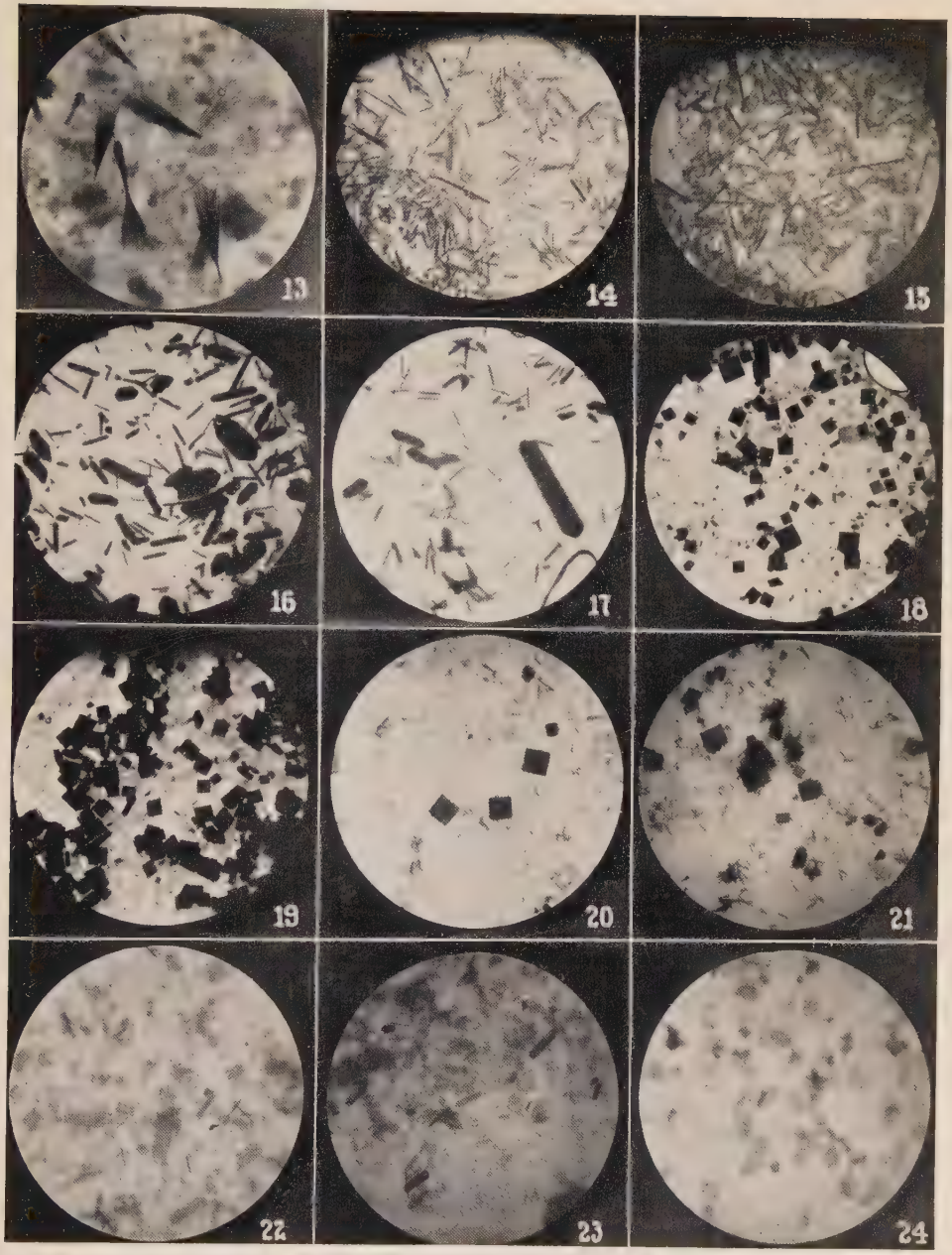
Fig. 13. Hog carbonmonoxide hemoglobin. × 18.6.
Fig. 14. Dog oxyhemoglobin. × 14.1.
Fig. 15. Dog oxyhemoglobin. × 14.1.
Fig. 16. Dog carbonmonoxide hemoglobin. × 62.
Fig. 17. Dog carbonmonoxide hemoglobin. × 62.
Fig. 18. Turkey carbonmonoxide hemoglobin. × 14.1.
Fig. 19. Turkey carbonmonoxide hemoglobin. × 14.1.
Fig. 20. Chicken carbonmonoxide hemoglobin. × 62.
Fig. 21. Chicken carbonmonoxide hemoglobin. × 62.
Fig. 22. Rat oxyhemoglobin. × 62.
Fig. 23. Rat oxyhemoglobin. × 62.
Fig. 24. Rat carbonmonoxide hemoglobin. × 62.

PLATE 5

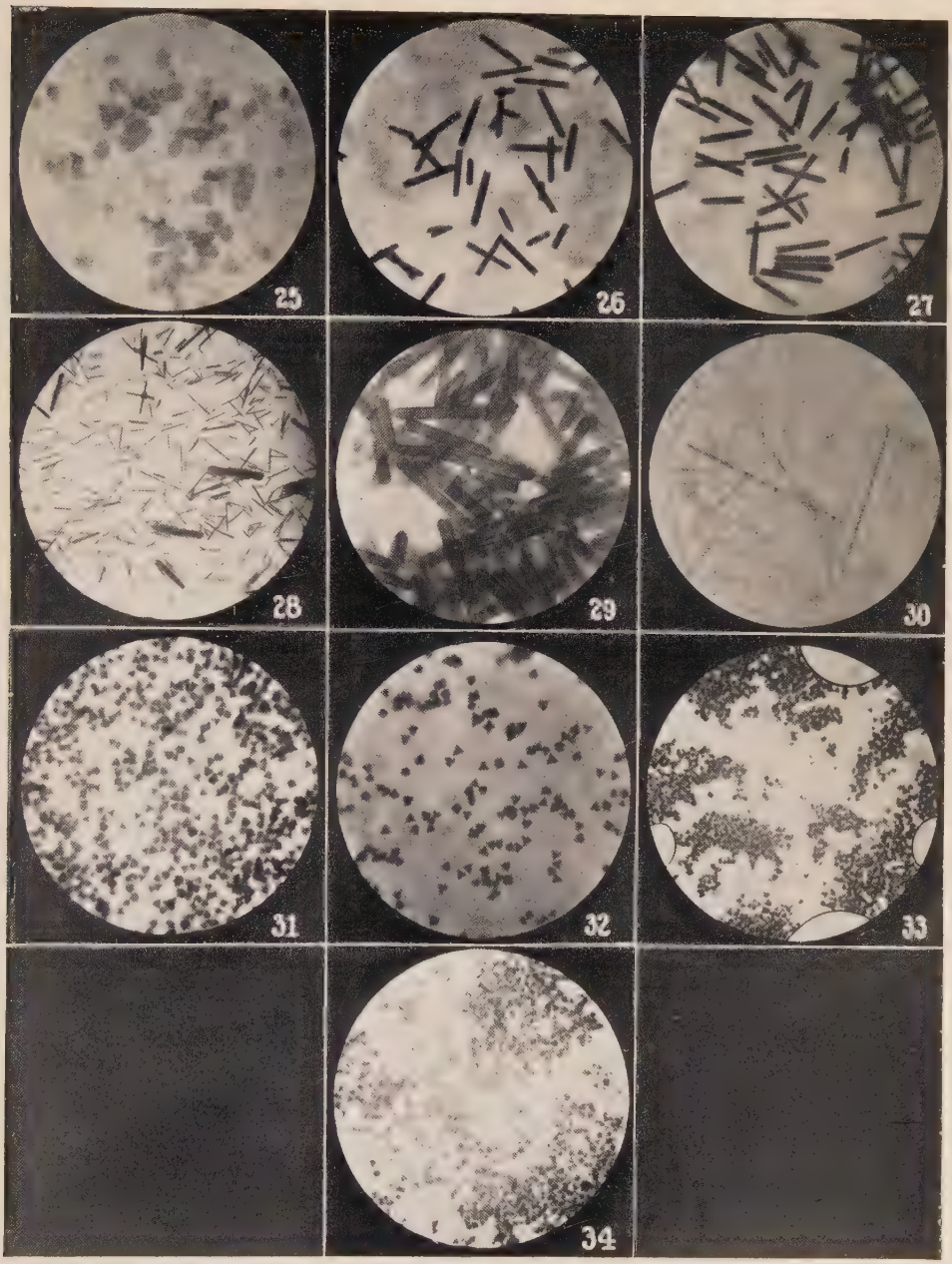
FIG. 25. Rat carbonmonoxide hemoglobin. × 62.
FIG. 26. Horse oxyhemoglobin. × 62.
FIG. 27. Horse oxyhemoglobin. × 62.
FIG. 28. Horse carbonmonoxide hemoglobin. × 14.1.
FIG. 29. Horse carbonmonoxide hemoglobin. × 188.
FIG. 30. Horse carbonmonoxide hemoglobin. × 62.
FIG. 31. Guinea pig oxyhemoglobin. × 62.
FIG. 32. Guinea pig oxyhemoglobin. × 62.
FIG. 33. Guinea pig carbonmonoxide hemoglobin. × 14.1.
FIG. 34. Guinea pig carbonmonoxide hemoglobin. × 18.6.



(Boor: Pure carbonmonoxide hemoglobin)



(Boor: Pure carbonmonoxide hemoglobin)



(Boor: Pure carbonmonoxide hemoglobin)

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A SPECTROGRAPHIC STUDY OF CARBON MONOXIDE HEMOGLOBIN.

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Plates 5 to 7.

(Received for publication, November 4, 1929.)

Carbon monoxide hemoglobin is species-specific. This fact has been shown recently by immunological methods (1). This specificity may be due to differences in the prosthetic group of the molecule of this conjugated protein, in the histone (globin) fraction, or in both the prosthetic group and the globin. The antigenic differences suggest, but do not conclusively prove that the globin fraction is the chemically species-specific part of the compound since this is the protein part and the largest portion of the molecule. It is presumed that the visible absorption spectrum of the carbon monoxide hemoglobin is due primarily to the effect of the colored prosthetic group. Since pure carbon monoxide hemoglobin was available, a study of spectra of this material presented itself, with the possibility that the species-specific portion of the molecule might be made evident.

In 1868 Preyer (2) showed the differences in visible spectrum between oxyhemoglobin, carbon monoxide hemoglobin, and other hemoglobin derivatives. Doumer and Fourrier (3) and Barcroft ((4) p. 41) have also compared the oxyhemoglobin and carbon monoxide hemoglobin spectra. In 1876 Sorby (5) measured the two main visible field absorption bands of human (normal) hemoglobin, and of blood of *Planorbis corneus*. He found them to be as follows:

	Center of band.	
	$\mu\mu$	$\mu\mu$
Normal oxyhemoglobin	581	545
Planorbis corneus	578	542½

Sorby concluded from this difference that in the *Planorbis* occurred, not the same oxyhemoglobin as in human, but a modification of it. Gamgee ((6) p. 131) in 1880 repeated the measurements with Preyer and found that "the position of the bands in *Planorbis* as stated above really coincides almost exactly with that of the bands of oxyhemoglobin." Welker and Williamson (7) found "that there is not sufficient difference in absorption coefficients of the hemoglobin of various species to serve as a means of identification of the species. This finding confirms the conclusions of most of the previous investigators." They used pure hemoglobin in their investigation. In 1922 Vlès (8) found considerable differences in spectra between the various species. Suhrmann and Kollath (9) gave some spectrophotometrical results on plasma and blood corpuscles of rat and blood corpuscles of guinea pig and man. Their results on the blood corpuscles of rat, guinea pig, and man indicated differences in absorption spectrum, especially between that of rat and man. However, this work was done on the mixture of the several blood proteins, lipoids, etc.; no pure hemoglobin was used. Barcroft (4), using a Hartridge reversion spectroscope, claimed differences in the spectra of both oxyhemoglobin and carbon monoxide hemoglobin from the different species.

The oxyhemoglobin and carbon monoxide hemoglobin, a study of which by spectrographic methods is herein reported, were prepared by a previously described method (1). This method was essentially as follows: Oxyhemoglobin was first prepared by the Marshall and Welker aluminum hydroxide cream method (10). Oxalated blood was centrifuged to procure the red corpuscles. This corpuscular mass was washed several times with salt solution and then laked with distilled water. Aluminum hydroxide cream was then added and the mixture filtered in a refrigerator. Marshall and Welker have shown that the filtrate contains no protein other than the oxyhemoglobin. The aluminum hydroxide cream used was made by adding slowly and with constant stirring, a 1 per cent solution of ammonium hydroxide to a 1 per cent solution of ammonium alum until the reaction was slightly alkaline. The creamy precipitate formed was then washed several times by decantation, the supernatant liquid finally giving a negative ammonia test with Nessler's reagent. Carbon monoxide hemoglobin was made

by saturating the oxyhemoglobin with CO gas which was generated by adding formic acid to warm, concentrated sulfuric acid. The gas was twice washed with sodium hydroxide and water. Both the oxyhemoglobin and carbon monoxide hemoglobin were then recrystallized from absolute alcohol at 0° or below. The concentrations of the materials were chosen as the result of a trial and error process. That concentration giving a clear, contrasting complete spectrum photograph in the time indicated in Figs. 1 to 7 was considered suitable.

In the work herein reported light from a Kromayer mercury arc lamp, when the ultra-violet spectrum was studied, or a 500 watt Mazda lamp, when only the visible spectrum was of interest was passed through quartz cells of 1 cm. thickness which contained the material under examination. A Hilger quartz spectrograph was used, spectra being photographed on Eastman orthochromatic and panchromatic plates.

All of the figures accompanying this paper are negatives; that is, the darkened portions of the plates are those areas affected by transmitted light. On all plates are one or two comparison spectra transmitted through empty cells and a series of spectra transmitted through the same cells filled with the material being studied. In Fig. 1 the dark lines represent the discontinuous mercury vapor spectrum, with or without the absorbing substance inserted. A comparison of the optical density of the corresponding lines gives the time of exposure necessary to restore the original intensity of that particular wave-length, although the absorbing substance is inserted. In this way the dotted curve in Fig. 1 was obtained, approximately representing a transmission curve. Figs. 2, 3, 4, and 6 were not used for such quantitative results, but rather to give evidence of small variations of transmission in nearly identical substances. In Figs. 5 and 7 the transmission is more evident, but still less quantitatively shown, on the background of a continuous spectrum from the 500 watt Mazda lamp, which, however, does not extend as far into the region of the shorter wave-lengths as 300 $\mu\mu$.

The approximate transmission curve of carbon monoxide hemoglobin is shown in Fig. 1, sheep carbon monoxide hemoglobin being taken as an example. The absorption bands appear as follows: the far ultra-violet end absorption occurs at 240 $\mu\mu$, the far ultra-

violet absorption at 280 $\mu\mu$, and the violet at approximately 400 $\mu\mu$. Visible absorption also occurs at about 540 and 570 $\mu\mu$, but this is made more evident by using the more continuous spectrum from electric light bulb source or by optical observation. It appeared of interest to include human carbon monoxide hemoglobin among the different species compared, but no crystallized human carbon monoxide hemoglobin was available. Absence of any spectrum differences between crystallized and non-crystallized horse carbon monoxide hemoglobin was shown (Fig. 2). Exposures of 1, 10, and 100 seconds were used. The spectra taken on the same photographic plate are the same, although the one material was recrystallized four times.

Fig. 3 shows spectra of human, sheep, hog, and ox carbon monoxide hemoglobin. The exposures are 10 and 100 seconds each, and comparison spectra of 1 and 10 seconds are also shown. No difference in spectrum of the different species is evident.

The relative stability of carbon monoxide hemoglobin and oxyhemoglobin was also investigated. On Fig. 4 are shown spectra of freshly made carbon monoxide hemoglobin of the same four species as shown on Fig. 3 and also material made by passing CO through human oxyhemoglobin which had been kept in the refrigerator at 0° for $\frac{1}{2}$ year, and human carbon monoxide hemoglobin made 12 days previously from fresh oxyhemoglobin. The spectrum of the material made from the half year old oxyhemoglobin shows considerable difference from wave-lengths 360 to 450 $\mu\mu$. Either the product of denaturation of the oxyhemoglobin by long standing is incapable of combining with CO to form carbon monoxide hemoglobin or a different combination of carbon monoxide and hemoglobin is produced. No change in carbon monoxide hemoglobin after 12 days standing was indicated by its spectrum.

The fact that oxyhemoglobin changes upon standing at room temperature is also shown in Figs. 5 to 7. A water solution of oxyhemoglobin was dialyzed in a collodion bag against distilled water at room temperature. After a few hours enough of a brown protein had passed through the bag to color the dialysate solution. Fresh oxyhemoglobin solutions are red, or in very dilute condition yellowish red. No red colored material passed through the collodion membrane. In Fig. 5 photographs are shown of the visible

spectrum of both fresh hog oxyhemoglobin solution and the brown dialysate exposed for 2, 5, 20, and 100 seconds. Comparison spectrum exposures of 1 and 10 seconds are also included. In Fig. 6 is a photograph of spectra produced by the ultra-violet lamp of the same materials as in Fig. 5. Protein content of the oxyhemoglobin and dialysate solutions is the same, in Fig. 6, as determined by the Kjeldahl method. Differences in spectra are evident at wave-lengths of 250, 436, and 578 $\mu\mu$.

It has been repeatedly stated ((6) p. 109 (11–12)) that oxyhemoglobin in water solution changes to methemoglobin upon standing. These statements were based mainly upon the changes in specific oxygen capacity and spectroscopic evidence. The spectroscopic evidence taken into consideration was mainly that the oxyhemoglobin modification showed an absorption band in the red at about the same position as that of methemoglobin and the two bands in the yellow and green of the oxyhemoglobin spectrum disappear as upon the addition of potassium ferricyanide. Fig. 7 shows the visible spectra of fresh oxyhemoglobin, oxyhemoglobin dialysate after 3 months dialysis, and methemoglobin,—all prepared from ox blood. The methemoglobin was prepared by the action of a few drops of saturated potassium ferricyanide solution on the oxyhemoglobin solution whose spectrum is shown. The dialysate of 0.12 per cent concentration is brown with a reddish cast. Its spectrum seems to be almost the same as that of methemoglobin of 0.08 per cent concentration. This is distinctly yellow. An absorption band in the red portion of the spectrum is seen in the spectroscope for both the oxyhemoglobin dialysate and the potassium ferricyanide methemoglobin. There is no discernible difference in position of this band, in the case of these two solutions. Fig. 7 shows the absence in the dialysate spectrum, as well as the methemoglobin spectrum, of the two bands, at approximately 578 and 545 $\mu\mu$, present in the oxyhemoglobin spectrum. The band beginning at about 398 $\mu\mu$ on the spectrum of oxyhemoglobin, and also methemoglobin, appears to be identical on the dialysate spectrum.

This comparison leads to the conclusion that oxyhemoglobin in water solution is unstable and changes to a brown substance whose molecular aggregates under these conditions are of a size and character which allow them to pass through the collodion membrane. This brown modification of oxyhemoglobin is partly

methemoglobin, the fraction increasing upon long exposure to water and oxygen. The dialysate spectrum of Figs. 5 and 6 seems to be more like oxyhemoglobin and less like methemoglobin than in Fig. 7. The dialysate represented in Figs. 5 and 6 was prepared by dialysis during a shorter time than in the case of the material used for making Fig. 7. It would be interesting to compare this material with the brown modification obtained by drying oxyhemoglobin and discussed by Barcroft ((4) p. 60) and Van Slyke, Hastings, Heidelberger, and Neill (13).

Although the color and spectrum of the dialysate were different from the original oxyhemoglobin, it gave a positive precipitin reaction against the antiserum formed by injection of rabbits with the oxyhemoglobin.

Ox and hog carbon monoxide hemoglobin were dialyzed for 5 weeks under an atmosphere of CO gas. The solutions were protected from all but subdued light. This caution was considered necessary because, as Hartridge (14) has shown, carbon monoxide is split off by sunlight and the freed hemoglobin is susceptible to change to methemoglobin. This is irreversible; *i.e.*, methemoglobin does not combine with carbon monoxide. Carbon monoxide hemoglobin and oxyhemoglobin change rapidly to methemoglobin under the influence of ultra-violet light. Biuret and precipitin tests showed that no protein appeared in the dialysate. Evidently, then, none of the carbon monoxide hemoglobin had changed to the brown modification of oxyhemoglobin found in oxyhemoglobin dialysate.

SUMMARY.

1. The transmission curve of carbon monoxide hemoglobin spectrum is shown.

2. Evidence of the purity of the carbon monoxide hemoglobin, made by the method described, is presented in Fig. 2. No difference is indicated between spectra of uncrystallized and crystallized carbon monoxide hemoglobin made by this method.

3. Spectra of carbon monoxide hemoglobin from different species are shown to be alike.

4. A water solution of oxyhemoglobin is shown to change upon standing; in time methemoglobin is formed, probably with another modification of oxyhemoglobin. Carbon monoxide hemoglobin seems very stable in this respect.

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EXPLANATION OF PLATES.

PLATE 5.

FIG. 1. Approximate transmission curve of sheep carbon monoxide hemoglobin, 0.08 per cent (Kjeldahl).

FIG. 2. Horse carbon monoxide hemoglobin, crystallized and not crystallized, 0.08 per cent (Kjeldahl).

PLATE 6.

FIG. 3. Carbon monoxide hemoglobin, not crystallized, 0.08 per cent (Kjeldahl).

FIG. 4. Carbon monoxide hemoglobin, not crystallized, 0.08 per cent (Kjeldahl).

PLATE 7.

FIG. 5. Hog oxyhemoglobin and its dialysate, equal concentration (colorimetric).

FIG. 6. Hog oxyhemoglobin and its dialysate, equal concentration (Kjeldahl).

FIG. 7. Ox methemoglobin 0.08 per cent, oxyhemoglobin dialysate 0.12 per cent, and oxyhemoglobin 0.08 per cent (Kjeldahl).

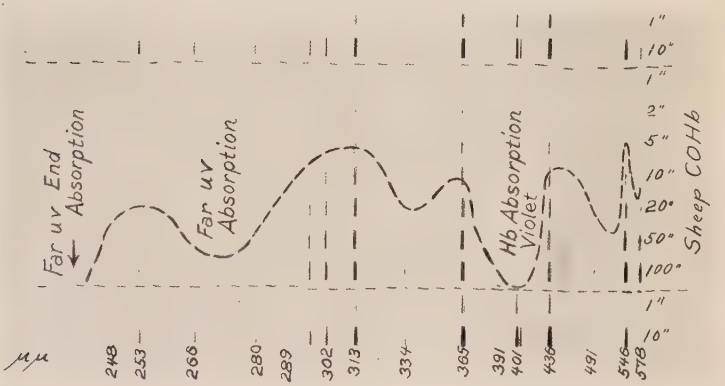
Far uv End Absorption
Far uv Absorption
Hb Absorption Violet
Sheep COHb
μμ
248
253
266
280
289
302
313
334
365
391
401
436
491
546
578
1"
10"
1"
2"
5"
10"
20"
50"
100"
1"
10"

FIG. 1.

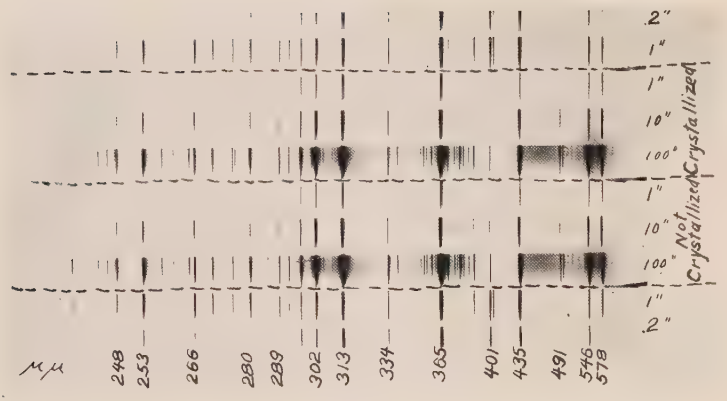
Not Crystallized
Crystallized
μμ
248
253
266
280
289
302
313
334
365
401
435
491
546
578
.2"
1"
1"
10"
100"
1"
10"
100"
1"
.2"

FIG. 2.

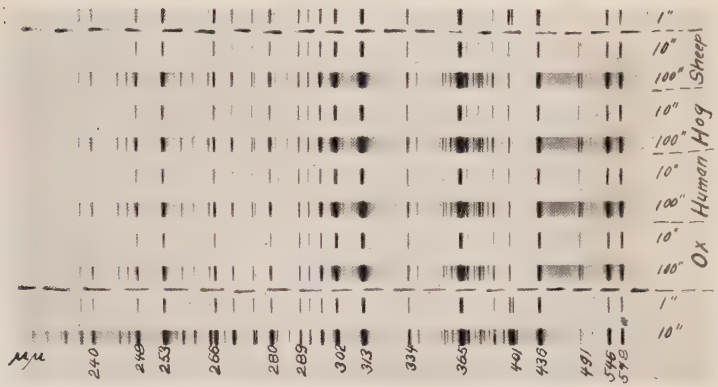

FIG. 3.

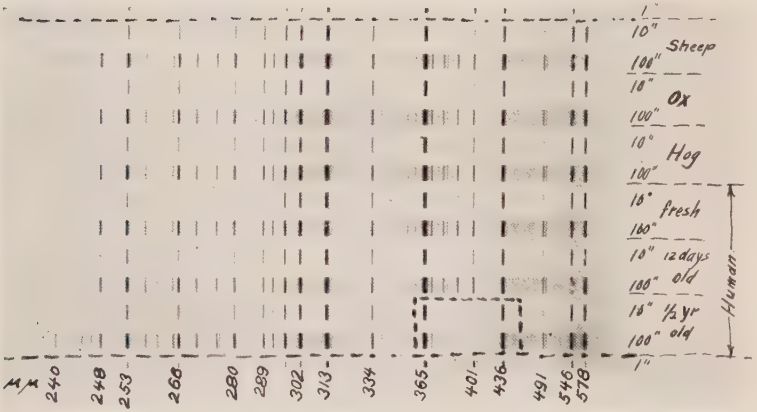

FIG. 4.

(Boor and Bachem: Carbon monoxide hemoglobin.)

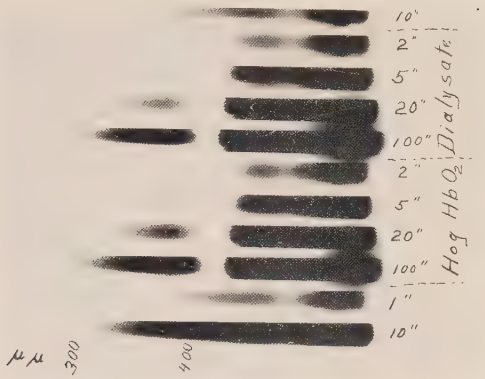

FIG. 5.

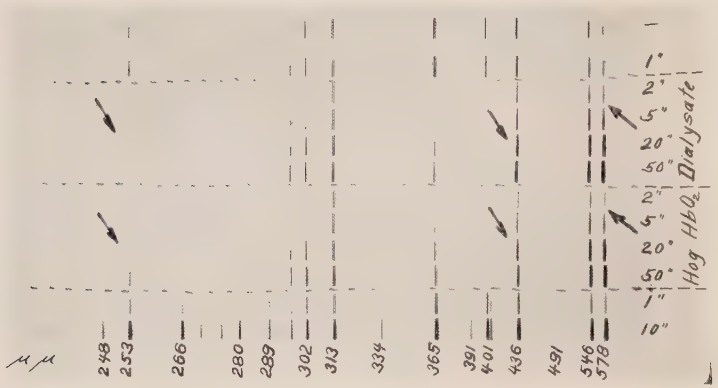

FIG. 6.

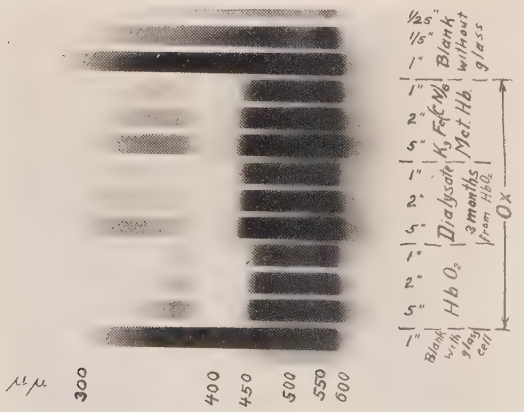

FIG. 7.

(Boor and Bachem: Carbon monoxide hemoglobin.)

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